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(54) Title: POLYMERIC FILM, ASSAY AND METHOD FOR DIRECT COLORIMETRIC DETECTION OF ANALYTES

(57) Abstract

A polymerized film, assay and method for direct detection of analytes using observable spectral changes in monomolecular films which occur upon the analytes selective binding to the film.

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POLYMERIC FILM, ASSAY AND METHOD FOR DIRECT COLORIMETRIC DETECTION OF ANALYTES

BACKGROUND OF THE INVENTION

Field of Invention

The present invention relates to a polymeric film useful in an assay and method for direct detection of small molecules, biomolecules and detectable analytes. The method and assay utilize observable spectral changes in monomolecular polymeric films, which changes occur upon the selective binding of a molecule, biomolecule or analyte to the polymeric film. The polymeric film comprises a lipid bilayer with an affinity ligand specific to the analyte, which layer responds to the binding of the analyte to the ligand by changes in light absorption spectra. The change is qualitatively and quantitatively detectable.

Background and Related Arts

Analytical chemistry methods for detection of most of chemical and biological molecules and/or analytes are virtually unavailable due to the destruction of the analyte characteristics during preparation and analysis steps and also because, typically, of the small amount of the analyte present in the test sample.

While useful in their own right, analytical chemistry methods are of limited or no practical applicability to many biological materials in which assessment would be valuable. Unless expensive and cumbersome gas chromatography methods are used, large quantities of analytes are generally required to accomplish detection. Often, quantitative results are either limited or not available.

Medical-biological systems analysis including direct microscopic observation using various cell-staining and classic pathology techniques also have limitations. Well developed microbiological techniques, such as culturing, colony characterization, and observation of metabolic and nutrient limitations are used to augment these techniques. While culturing and direct tissue observation techniques have served

as the bulwark of medical detection processes for many years, they also have considerable limitations. Pathological analysis of patient tissues to determine the stage of development of a disease and the identification of the causative pathogens generally requires an invasive procedure. On the other hand, culturing the pathogen from various body fluid or other samples is time consuming and expensive.

A breakthrough in medicine occurred with the development of immunoassay methods. In these methods, an antibody is developed which specifically binds to a target of interest. While costly in both their development and production, antibodies from animals typically allow a very accurate analysis of a number of analytes which had previously been virtually unassessable in both research and clinical situations.

An important technical advancement in immunoassay was the development of monoclonal antibodies. Because the antibody itself is a small molecule, it is preferably labeled in some way so that the binding event can be detected. This can be done with a dye, fluorescent, radioactive or other label. Conversely, if binding inhibition occurs between a known amount of introduced, labeled analyte and the material to be analyzed, the diminution of the signal indicates the presence of the test analyte. If the agglutination of the antibody particles is of sufficient volume and density, the formation of a precipitant can also serve to signal the presence of an analyte.

In recent years, the research and medical communities have come to rely heavily on immunoassay techniques to detect and quantify biological materials. While successful in many respects, the indirect nature of immunoassay methods as well as their dependence on antibody materials results in a variety of complications, problems, and assay limitations. The development and production of antibodies remains expensive, and these molecules are sensitive to environmental changes. Also, only those materials to which antibodies can be produced can be detected by these systems.

Many small biological molecules are notoriously difficult

to assay in a direct manner due to the severe limitation of environmental ranges which they can tolerate without losing their specific characteristics. For these molecules, immunoassays have been heavily relied upon. The requirement that an antibody be developed and produced for each possible target limits the efficacy of immunoassay methods in such applications as designer drug development and screening.

A disadvantage of immunoassay systems is readily apparent in rapidly evolving pathogens such as the influenza virus, where the external coat of the pathogen which is normally available for immune reactions constantly shifts its antibody recognition elements and, therefore, becomes immunologically unrecognizable.

Certain types of analytical chemistry techniques were optimized by the immobilization of one or more of the components of a reaction. For instance, if the material to be tested is present in only a small quantity in a test sample, the detectable analyte may be at so small a concentration that it is beyond the detection capabilities of any normal assay system. In these instances, immobilization systems have proven to be advantageous.

Many immobilizing materials such as Sephadex columns are available. Requirements for these materials are their specific binding properties, their relatively inert reaction to other materials so that they themselves do not interact in the test reaction or otherwise interfere with the assay for immobilization, and their structure regularity providing predictability in the testing situation.

Classically, immobilization has been accomplished on columns, liposomes or other surfaces. The use of such materials provides many advantages for an assay system. For instance, these materials allow easy segregation of reactants from the other sample components.

In a typical immobilization scheme, the analyte is concentrated by adhesion to the immobilizing material for which it has a specific affinity. The testing then takes place on an area surface limited to immobilized surface, rather than in

the defused three-dimensional array of the original sample fluid. The results are concentrated in a smaller area, and are more likely to be detected.

Bilayer films emplaced on surfaces have been used to provide the immobilization matrixes. Chemical modifications of these surfaces by organic monomolecular films have recently been used in an effort to develop new materials. The techniques of molecular self-assembly, such as that described in Langmuir, 3, 932, (1987) are used for coating surfaces with a well-defined, quasi two-dimensional array of molecules. These bilayer films became useful as immobilizing supports for analytic reactions. Bio-sensors based on these films can detect molecules of diagnostic significance such as glucose and urea, as described for example in Thin Solid Films, 180:65, (1989) and 210: 443 (1992). In these cases, classic analytical chemistry systems are immobilized on the films in order to improve the readout of the test results and otherwise simplify and improve the detection capabilities of the test procedure.

Similarly, the detection of receptor-ligand interaction is accomplished by indirect assays such as the enzyme-linked immunosorbent assay. Although biotechnological functionalized films have led to molecular recognition at an interface, the problem of translating the molecule recognition event into a measurable signal has remained problematic until the advent of the subject invention.

Detection of the immobilized reaction products is generally carried out by coupling the immobilized matrix to a secondary device such as an optical fiber (Colloid Interface Sci., 124: 146 (1988)), quartz oscillator (Thin Solid Films, 210: 471 (1992)), or electrode surfaces (Chemical Letters, 627 (1990)).

Some of the analytes bound to the immobilized matrixes are detectable by the fluorescent label, where the fluorescence or its quenched state indicate the occurrence of a binding event. Immobilized matrixes may further be made of a bi-lipid layer where the detection materials are embedded in the surface of the supporting bi-lipid layer (Advanced Materials, 3: 532

(1991)).

Polydiacetylene films are known to change color from blue to red with increasing temperature or changing pH due to conformational changes in the conjugated backbone (Langmuir, 8: 594 (1992)). While it has been a goal of the research community to exploit this characteristic in the detection of binding events, researchers have yet to develop a method using this phenomenon in practical applications.

It would be, therefore, highly desirable if the direct detection method could be provided for detection of very small chemical and biological molecules present in minute amounts. It would be ideally advantageous to develop technology of monomolecular film supports in a simple and unique way so that the binding would event cause a readily observable change in the support material that could be directly detected.

It is therefore, a primary objective to provide a simple and reliable assay for detection of minute amounts of various chemical and biological analytes using a novel polymeric film for direct colorimetric detection of analytes.

SUMMARY

It is one object of the present invention to provide a polymeric film, useful in an assay for detection of minute amounts of chemical or biological analytes, a method and assay for direct colorimetric detection of small molecules of various analytes using observable color changes in a monomolecular polymeric film occurring when the molecule binds to a ligand of the film polymer bilayer.

It is another object of the present invention to provide an assay suitable for detection of the presence of minute amounts of chemical or biological molecules by directly detecting the binding event when the analyte specifically binds to a ligand of the polymer bilayer.

It is a further object of the present invention to provide a method and assay for a direct detection of viruses, bacteria, parasites, and other pathogens, or drugs, hormones, cell wall fragments, enzymes and their interactions, as well as other biologically relevant materials.

It is another object of the present invention to provide a method and assay for the development of drugs and improvement of drug activity by observing competitive binding or inhibition of natural binding events between some or all surfaces of the polymeric film and the natural bioactive ligand of the tested biomolecule.

It is yet another object of the invention to detect the presence of biomolecules, using visible color changes or colorimetric detection in the lipid bilayer of the polymeric film of the invention which color changes occur as a result of the specific binding of the biomolecules to the bilayer.

It is an additional object of the present invention to provide a simple to use, inexpensive and reliable assay and a test kit for qualitative and quantitative detection of minute amounts of small molecules, which assay is stable in a wide range of environmental and laboratory conditions when the analyte is mixed with a number of other materials.

It is still another object of the present invention to provide a polymerized bilayer film for direct detection of the presence of analyte comprising:

a) a ligand having direct affinity for the analyte or which functions as a competitive binder to the analyte;

b) a linear structural linker having two terminal ends, wherein said linker is attached at its first terminal end to said ligand;

c) a conjugated polymer backbone to which said structural linker is bound at its second terminal end;

d) orienting head groups bound to the surface of the conjugated polymer backbone in positions not occupied by the structural linker; and

e) a support structure;

wherein said film undergoes detectable spectral modification upon binding of a target analyte to the ligand moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a schematic view of one embodiment of the subject invention.

Figure 2 is an optical micrograph of one of the inventive polymeric films (Figure 2A) and the optical micrograph illustrating the colorimetric response to the influenza virus (Figure 2B).

Figure 3 shows the structures of the compounds used in film formation tested in competitive inhibition experiments.

Figure 4 illustrates quantification of the visible absorption spectrum before and after incubation with virus.

Figure 5 is a plot of the colorimetric response of a sialoside bilayer film to increasing concentrations of an influenza virus.

Figure 6 illustrates usefulness of the invention for detection of the influenza virus by detecting colorimetric response in the presence or absence of the binding inhibitor.

DEFINITIONS

As used herein:

"Analyte" means a detectable chemical molecule, biomolecule or a portion thereof which is detectable by a specific binding to a ligand of the polymeric film of the invention, resulting in changes in the spectral characteristics of the polymeric film.

"Ligand" means a hydrophilic lipid monomer or its derivative to which the tested analyte binds at a specific recognition and detection site, which can be rendered polymeric by linking the ligand through a linking arm to a polymerized thin bi-layer film. The ligand is typically specific either to the individual analyte or to a group thereof. The ligand forms a part of detecting heads of the polymeric film. The ligand is attached to one terminal end of spacer or linker molecules and is polymerizied in admixture with a hydrophobic matrix lipid monomer. The ligand can be monovalent or multivalent.

"Linker" or "spacer" means a linear structure molecule linked through one terminal end to the ligand and through the second terminal end to the base film. Specifically, the linker is attached to one of several monomers which have been polymerized into a chromatic detection element. A structural

linker has a sufficient length and conformability to allow binding of multiple sites on the analytes. The linker may be, for example, tetraethylene glycol.

"Conjugated polymer", "polymer backbone", "conjugated polymer backbone" means a layer of a polymerized ligand and matrix monomer bi-layer assembly able to signal binding occurring at the surface of the film by a chromatic transition. The polymer backbone may be, for example, polydiacetylene. The polymer backbone is bound to a structural linker.

"Polymer bi-layer" is made of polymerizable ligand and matrix lipid monomers, polymerized into a chromatic detection element. Binding of the analyte to the ligand linked to the polymer backbone induces stress within the bi-layer, changing the effective conjugation length of the polymer backbone. The matrix lipid monomers suitable for polymerization are lipid monomers. Such moieties include: acetylenes, diacetylenes, alkenes, thiophenes, imides, acrylamides, methacrylates, vinylether, malic anhydrides, urethanes, allylamines, siloxanes or vinylpyridinium etc. Lipids containing these groups can be made into homopolymers or mixed polymers. The preferred group for use in this invention is diacetylene, due to its unique optical properties in the polydiacetylene polymerized form. However, other polymerizing groups could be used when they provide an observable change in properties upon a binding event.

"Ligand lipid monomers" are carbohydrates, amino acids and such other molecules having carboxylic groups.

"Lipid monomers" are readily polymerized into polymeric films by ultraviolet irradiation or other means for polymer backbone formation. Most preferred monomers are diacetylene monomers such as polydiacetylene and octadicyltrichlorosilane.

"Lipid orienting head groups" means lipids forming, stabilizing and structurally orienting the polymer bi-layer to facilitate color stability until the binding of the analyte disturbs it.

"Lipid tail(s)" means a moiety serving to anchor the polymerized film to the support surface.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns an assay and method for the direct colorimetric detection of small molecules, biomolecules and analytes binding in a receptor-ligand-like interaction using a novel polymeric thin film construct. The invention further concerns the thin polymeric film as well as the method for producing the polymeric films for specific detection of various analytes through their binding to a ligand, where the ligand or its derivative are rendered polymeric by polymeric linking of the ligand through a linking arm to a polymerized thin bi-layer film.

The presence of the investigated analyte detected by binding of the analyte to the ligand is observed through changes in the spectral characteristics of the polymeric film. The polymer-ligand assembly encompasses specific molecular recognition and detection sites, all contained within a single molecular assembly.

The present invention allows, for the first time, direct detection of small chemical and biological molecules, such as pathogens and drugs, using observable spectral changes in monomolecular films. The present invention thus represents an entirely new approach to the direct detection of small molecules, biomolecules and analytes using color changes in a monomolecular film which changes occur when these materials specifically bind to the target molecule.

The subject invention represents a dramatic advancement over both chemical and immunoassay systems as it enables both qualitative and quantitative detection of small, otherwise undetectable, chemical molecules, biomolecules and/or identifiable analytes in a simple, fast and practical way. The present invention combines the advantages of both immunoassay and chemical analysis in a single system.

Additionally, the assay of the invention is useful for environmental testing by detection of various analytes in their most advantageous environmental conditions by the allowing rigorous, direct analysis to occur even in very narrow environmental ranges. The speed and simplicity of the color

change indicator of the subject invention are its hallmark advantages.

In general, the present invention requires no pre-analysis purification step. The results are easily read by an untrained observer, and the test can be conducted in ambient conditions. Very mild testing conditions allow the detection of small biomolecules in a near natural state, providing information as to their interactions and avoiding the risk of modification or degradation of the analyte. This feature of the subject invention is due to the high specificity of the ligands incorporated into the detecting film. Additionally, the inventive direct assay system avoids the expense, complications, and increased inaccuracies inherent in the indirect systems currently available.

I. The Polymerized Bilayer Film

The polymeric film according to the invention is a multi-layer assemblage which allows for the direct detection of the presence of a wide range of analytes by changes in color spectral criteria.

A. Preparation of Polymer Film

Polymeric detection films of the invention are simple and easy to prepare.

Briefly, monomeric lipids having hydrophilic groups, such as carbohydrates, amino acids, etc. which groups are able to bind to biological materials, are used as the ligands for detection of these materials. Monomeric matrix lipids, having hydrophobic groups, are used to assemble and orient the film. Both lipids are mixed in proportion from about 5-40% of ligand/matrix lipids in an organic solvent, such as chloroform, benzene, hexane, xylene, and others, or in a mixture of the organic solvent with an aqueous solvent, such as a mixture of chloroform/methanol, preferably about 9:1. The solution of ligand/matrix lipids in the solvent is slowly added, typically in small drops, onto the surface of the water, where it orients itself by attaching the hydrophilic groups to the water and by projecting the hydrophobic groups out of the water. During this step, the organic solvent either evaporates or is removed

leaving behind the lipids as a fluid film positioned in the water surface. For better film formation and performance, the lipids are either applied in high concentration on small water area or, typically, are compressed in sandwich-like manner by placing barriers on both sides of the lipid film. Then, the lipid monomers are polymerized by, for example, exposure to the UV light forming thereby a floating polymer which is, to a naked eye, visibly blue. This polymer is then mounted on, or attached to, the separately prepared support structure, such as, for example, glass, microscopic glass, celluloid, etc., or any other firm supporting structure which can be coated with an anchoring hydrophobic surfactant such as octadicylsiloxane, or hexamethyldisiloxane, and others, wherein the siloxane group provides anchoring. The final assembly has blue color.

The inventive diacetylenic lipid monomers such as compound 1 (Fig. 1) are readily polymerized in monolayers by ultraviolet irradiation to form a conjugated polydiacetylene backbone of alternating eneyne groups using standard art methods (Colloid Polymer Science, 255: 36 (1977), and J. of Polymer Science, Letters to the Editor, 16: 205 (1978)). In one embodiment of the invention, a thermo-chromatic polydiacetylene bilayer is assembled on a support, and then used for the detection procedure. The polydiacetylene layer is functionalized with a receptor specific ligand for the target molecule which is to be detected. Both qualitative and quantitative findings as to the presence of the target material can be obtained using various embodiments of the subject invention.

In one embodiment of the present invention, the bilayer is composed of a self-assembled monolayer of octadecyltrichlorosilane and a Langmuir-Blodgett monolayer of polydiacetylene (Langmuir-Blodgett Films, Roberts., Ed., Wiley New York, (1966)). The polydiacetylene layer in this case is functionalized with an analog of sialic acid. Sialic acid is the receptor-specific ligand for the influenza virus hemagglutinin, as well as for other pathogens. The sialic acid ligand serves as a molecular recognition element.

This colorimetric technology is coupled to materials whose

chemical properties can be tailored to bind a variety of small organic molecules. Many organic hosts form inclusion complexes with dipolar protic and aprotic compounds. Certain inclusion compounds, or clathrates, such as compounds 1 and 2 seen in Reaction Scheme 1 have been shown to be highly selective sorbents for organic solvent vapors (Angew. Chem. Int. Ed. Engl., 32: 110 (1993)). For example, compound 1 has a pronounced affinity for dioxane and little affinity for butanol, acetone, methanol, 2-propanol, cyclohexane, toluene and water. The lack of affinity to cyclohexane is particularly remarkable given the similarity in chemical structure. Compound 2 on the other hand, shows a pronounced affinity for 1-butanol over the same group of solvents. This breakthrough, combined with colorimetric detection led to a new class of chemically sensitive materials immobilized on surfaces. Surfaces which have both the clathration element and the detection element built into a single supra-molecular assembly represent a novelty of the method for direct detection of a wide variety of environmental contaminants.

Clathrate-forming compounds coupled to the polydiacetylene polymer form a new class of materials which are chemically sensitive, robust, and have unique optical properties. These materials offer a novel, yet simple method of detecting the presence of organic solvents by monitoring the color changes which occur in the film upon binding of the offending compound. No technical expertise is required to use such a detector; thus it is suitable for on-site analysis by persons with little or no technical experience. The molecular level understanding of why clathrate-forming compounds of a given structure complex with a given test molecule leads to a wide variety of clathrate-forming polymeric thin films. All these are within the scope of the invention.

B. The Composition of the Polymeric Film

The inventive assay film is composed of the base polymeric bi-layer film, whose surface contains both orienting and detecting head groups. The detecting head groups are composed of a ligand specific to the analyte in question, which is bound

to one terminal end of a linear structural linker. This linker, in turn, is bound to the base film by its second terminal end. The base film surface is also provided with lipid orienting head groups.

A schematic depiction of one embodiment of the present invention is seen in Figure 1.

Receptor-binding lipid ligand (1) which is hydrophilic in nature is shown attached through the terminal end of its linker or spacer molecule (3) to the second lipid matrix monomer of the polymeric film which is hydrophobic in nature. Both the ligand and matrix lipid are polymerized into a chromatic detection element (5). The chromatic detection element (5) which is, through its hydrophobic side, connected to a monolayer support layer (7) which is also hydrophobic and is, in turn, attached to a support structure, such as a microscopic slide (9). Alternatively, any surface which will accommodate the hydrophobic detection element (5) can be substituted for the elements (7) and (9). For example, a plastic surface could serve in their place.

The polymeric film comprises a ligand lipid monomer, optionally a compound used as a linker or spacer, and a matrix lipid, both polymerized into the detection polymeric film having detection and orienting head groups, said film mounted on the support system.

1. Ligand Group

A ligand or its derivative is a hydrophilic lipid monomer to which the tested analyte binds at a specific recognition and detection site, which can be rendered polymeric by linking of the ligand through a linking arm of the linker or spacer to a polymerized thin bi-layer film. The ligand is typically specific either to the individual analyte or to a group thereof. The ligand forms a part of the detecting heads of the polymeric film and it can be monovalent or multivalent.

The ligand group of the present invention, seen in Figure 1 (1) is selected from a wide variety of materials. The main criteria for such selection are that the ligand has an affinity and specificity for the analyte of choice. The ligand may be

directed to a single entity or to a broad range of materials, such as when a class of materials is to be assayed. Appropriate ligands include peptides, carbohydrates, nucleic acids or any organic molecules which bind to receptors. For instance, all influenza strains use the same binding sites when binding to a host receptor molecule. Thus, the host receptor molecule is advantageously employed for screening for all influenza strains, including those which have not yet been characterized.

Ligands are also advantageously used in the present invention when they function as competitive binders to the analyte, for instance, when a pathogen is introduced with a test material which is to be tested for the presence of a receptor molecule. In absence of the receptor molecule, the pathogen binds to the assay bilayer and produces a color. To the degree that the pathogen surface binds to the receptor molecule introduced in the test material, the binding is diminished. In this way, the presence of a receptor molecule is detected and quantified.

2. Linker

A linker or spacer may be a separate linear structure molecule linked through one terminal end to the ligand and through the second terminal end to the base film or a part of the ligand or matrix. Specifically, the linker is attached to one of several monomers which have been polymerized into a chromatic detection element. A structural linker has a sufficient length and conformability to allow binding of multiple sites on the analytes. The linker may be, for example, tetraethylene glycol.

3. Polymer backbone

Polymer backbone is a layer of a polymerized bi-layer assembly able to signal binding occurring at the surface of the film by a chromatic transition visible as a change of color from blue to red. The polymer backbone may be, for example, polydiacetylene. The polymer backbone is bound to polymer bi-layers.

Polymer bi-layer is made of polymerizable monomers,

preferably ligand/matrix lipids polymerized into a chromatic detection element. Binding of the analyte to the ligand linked to the polymer backbone induces stress within the bi-layer changing the effective conjugation length of the polymer backbone.

The monomers suitable for polymerization are lipid monomers. The matrix lipid monomers are such moieties as: acetylenes, diacetylenes, alkenes, thiophenes, imides, acrylamides, methacrylates, vinylether, malic anhydrides, urethanes, allylamines, siloxanes or vinylpyridinium etc. Lipids containing these groups can be made into homopolymers or mixed polymers. The preferred group for use in this invention is the diacetylene group due to its unique optical properties in the polydiacetylene, its polymerized form. The most preferred matrix monomers are diacetylene monomers such as polydiacetylene and octadicyltrichlorosilane, however, other polymerizing groups could be used when they provide an observable change in properties upon a binding event. The second component of the bi-layer are the ligands described above.

Lipid monomers are readily polymerized into monolayers by ultraviolet irradiation or by other means.

4. Lipid Detecting and Orienting Groups

The lipids are important in forming and structurally orienting the film's bi-layer so that binding of the analyte results a detectable color change. The detection is based on a structuring effect of the orienting groups appropriately stabilizing the physical structure of the bi-layer to facilitate color stability until the binding of the analyte to the molecular recognition ligand groups happens. The binding causes sufficient steric perturbation or stress of the structure to result in a color change. The stability and relative rigidity engendered by the orienting lipids unites the bi-layer, so that a steric change in one area triggers a larger effect in the surface as a whole which can be readily observed.

The observed spectral changes are due to stresses induced by binding which change the effective conjugation length of the

polymer backbone.

Materials, suitable as ligand detecting groups in the present invention are hydrophilic lipids including $-CH_2OH$, $-CH_2OCONHPh$, $-CH_2OCONHEt$, $-CH_2CH(Et)OCONHPh$, $-(CH_2)_9OH$, $-CH_2OCOPh$, $-CH_2OCONHMe$, $-CH_2OTs$, $-CH(OH)Me$;

$-CH_2OCOR_2$, wherein R_2 is n-C_5H_{11}, n-C_7H_{15}, n-C_9H_{19}, n-$C_{11}H_{23}$, n-$C_{13}H_{27}$, n-$C_{15}H_{31}$, n-$C_{17}H_{35}$, Ph, PhO, or o-$(HO_2C)C_6H_4$;

$-OSO_2R_2$, wherein R_2 is Ph, p-MeC_6H_4, p-FC_6H_4, P-ClC_6H_4, p-BrC_6H_4, p-$MeOC_6H_4$, m-$CF_3C_6H_4$, 2-$C_{10}H_7$, or Me; and

$-CO_2M$, wherein M is K,HNA, or Ba/2.

The preferred materials which can be employed as head groups in the present invention are:

- $CH_2OCONHR_2$ or $-CH_2CONHR_2$ where R_2 is Et, n-Bu, n-C_6H_{13}, n-C_8H_{17}, n-$C_{12}H_{25}$, cyclo-C_6H_{11}, Ph, p-MeC_6H_4, m-MeC_6H_4, o-ClC_6H_4, m-ClC_6H_4, P-ClC_6H_4, o-$MeOC_6H_4$, 3-Thienyl, Me, Et, Ph, 1-$C_{10}H_7$, Et, Ph, $EtOCOCH_2$, $BuOCOCH_2$, Me, Et, i-Pr, n-C_6H_{13}, $EtOCOCH_2$, $BuOCOCH_2$, Ph, or 2,4$(NO_2)_2C_6H_3OCH_2$, CH_2CH_2OH.

The most preferred detection groups are taken from $-CH_2COX$, where X is OH, MeO or any salt thereof.

Materials suitable for use as matrix orienting groups are hydrophobic lipids used to assemble and orient the film. The groups comprising the tails of the lipids are of a wide variety. Serving to anchor the polymerized film to the support surface these moieties can be any of the following: CH_3-, CH_3O-, neo-$C_5H_{11}O-$, cyclo-$C_6H_{11}O-$, $PhCH_2O-$, p-AcC_6H_4O-, p-BzC_6H_4O-, p-$BrC_6H_4COCH_2O-$, p-$(PhCH=CHCO)C_6H_4O-$, p-$(PhCOCH=CH)C_6H_4O-$, o-BZC_6H_4NH-, p-BZC_6H_4NH-, $MeOCH_2CH_2NH-$, n-$C_6H_{13}NH-$, EtO-. The preferred group in this invention is the methyl group.

5. <u>Film Support</u>

The supporting structure (9) to which the film is attached can be a variety of materials. The material used in certain embodiments of the invention, such as Example 1 is a microscopic glass slide which has been made hydrophobic by treatment with an appropriate surfactant such as octadecyltrichlorosilane. Any material which is somewhat hydrophobic such as plastic, mica metal, ceramic or other relatively uniform polymeric surface can be used. Glass is the

preferred transferrant support in this invention due to its transparency for ease in reading the color changes. However, non transparent materials can be employed using a reflectance type measurement.

B. Receptor-Binding Molecules Used as Ligands

Receptor-binding molecules are materials on the surface of a host cell to which a pathogen attaches itself as a prelude to the infective event. Selecting such molecules as the ligand group of the present invention provides a specific recognition site for these pathogens as these molecules tend to be highly genetically conserved in the pathogen having obvious criticality to the pathogen survival. Different strains of the same pathogen will generally not produce a false negative result when molecules such as the ligand group in the subject invention are selected. Receptor molecules tend to be smaller and less complex, and often less hydrophobic.

Receptor-binding molecules such as those described above are detectable by the current invention as an increasing number of receptor molecules have been recognized, identified, isolated, and synthesized for a large number of pathogens. Many of these receptors have been improved for use in various analytic and treatment systems.

A good example of the usefulness of the invention is the sialic acid derivative used for detection of influenza or malaria. Examples of the receptors for a number of pathogens are provided in the application as Table 1. All of these, as well as any other ligands falling within the scope of the invention are within the scope of the subject invention.

Example 1 describes one exemplary use of sialic acid derivatives as one preferred embodiment for the use of receptor-binding molecules.

II. Assay Testing Conditions

The assay of the invention is performed under gentle testing conditions sensitive to tested analytes. The inventive polymeric thin film construct employs ligands and analytes which are stable or possess appropriate binding characteristics specifically within a limited *in vitro* or narrow environmental

range of conditions. The present invention meets stringent limitations even within this narrow range of such conditions. This allows, for instance, maintaining three dimensional conformations of sensitive biochemicals and biomolecules throughout the testing procedure.

The test is extremely simple in that the tested analyte or molecule is contacted with the polymeric film of the invention and the color change and its intensity are observed and measured for quantitation. Typically, this process lasts only about 30 minutes.

The present invention functions well even in extremely limited conditions. The assay conditions, such as pH, salinity, and temperature can be carefully controlled by feedback controls, titration and other techniques without interfering with the accuracy or sensitivity of the analysis.

Because of this wide experimental range advantage of the present invention, intact cells or sensitive subcellular inclusions are assayed without disturbing their structural integrity. Subtle cellular development stages, such as the various stages of malaria infection can be monitored. Additionally, the association between various factors can be tested or monitored even during the interaction process using the method of the subject invention.

The inventive film and method are suitable for assaying of very small biological or other molecules for which antibodies cannot be developed. These target materials include organic solvents or pollutants present at extremely low levels. There are special opportunities made available by the advances achieved by the subject inventors for drug screening in both forensic and clinical applications. Inhibition techniques applied to the subject invention allow the testings of materials which are of a minute size or have a small number or single valiancy.

The unexpected spectral signal achieved by the present invention is due to a physical perturbation of the bilayer which occurs as a result of the binding event of for example, multivalent materials, such as viruses and cell membrane

fragments, which perturbation then is detected using the subject inventive method. Thus, multivalent materials generally elicit a particularly strong response in the assay system because of conformational changes introduced into the bi-lipid layer as a result of binding, causing physical reconfiguration of structure.

Pre-binding of smaller, single valent analyte materials to a carrier may also prove advantageous to increasing the efficacy of the subject invention. For instance, the analyte may be bound to a polymer or the surface of a liposome. This would concentrate the binding event on the inventive bi-lipid surface to specific points, increasing the spectral modification at each point of contact. Additionally, the curved surface of the liposome to which the analyte is attached would serve to tug the peripherally bound analytes away from the bi-lipid surface and force analytes centrally located on the liposome into the bi-lipid surface. This pre-binding step then results in increased torsion, perturbation and signal generation on the bi-layer surface.

The assay of the invention is suitable for detection of weak binding analytes as well as for multivalent analytes. The multivalent feature of the polymer-linked ligands of the subject invention provides a heightened binding capacity in the case of naturally multivalent analytes. Multivalency can also be provided for limited valency analytes prior to the test procedure to imbue them with this advantage of the subject invention. The inventive exploitation of multivalency allows a specific but weak interaction to be amplified many fold.

A structural linker of sufficient length and conformability aids the binding of multiple sites on the analyte even when they are conformationally separated on a curved surface. As a result of these special features, the present invention can detect many ligands previously unsuitable for assay evaluation.

The main criteria for effective indication of the presence of an analyte is that the surface of the indicating bi-layer be sufficiently perturbed to produce the requisite spectral

change. Binding the analyte to an immobilizing particle serves this purpose, as it concentrates the analyte in a small area, and further provides a three-dimensional aspect over a relatively large area to even a small analyte.

A large variety of ligands may be employed in the subject invention, allowing great flexibility in detecting a multivalent test target. Ligand selection is based on the most advantageous binding and steric characteristics, rather than on accommodation of the test system. Thus, the most advantageous ligands are selected based on such factors as hydrophobicity and hydrophilicity, size, position of binding site, and conflicting affinities vis-a-vis the analyte to be detected. Ligands which are advantageously employed in the subject invention include carbohydrates, peptides, nucleotides, heterocyclic compounds, and other organic molecules.

The inventive polymerized bi-layer assemblies film structure and morphology are shown in Figure 1 as a schematic diagram of the polymerized bi-layer assembly. The siloxane linkages of the bottom monolayer are not shown. Fig. 2 (A) shows an optical micrograph of the sialoside bilayer assembly between crossed polarizers. Large domains up to 150 μM are visible. Scale: 1 cm = 20 μM.

The initial investigations focused on the binding of the influenza virus to sialic acid as a model system for colorimetric detection. The study is reported in Example 1. A lipid monomer contains a carbon-linked sialic acid head group that provides a molecular recognition site for the viral lectin, hemagglutinin. Figure 3 shows the matrix lipid (11) sialoside lipid (13) and lactose lipid (15) used in LB film formatting and compounds α - NeuAc (17), β-O-NeuAc (19), and α glucose (21) used for competitive inhibition experiments as described in Example 4. The syntheses of compound (13) is reported in J. of The American Chemistry Society, 115: 1146 (1993). A carbon glycoside was used instead of the naturally occurring oxygen glycoside to prevent hydrolysis by the neuraminidase, which is also present on the surface of the virus.

III. Target Materials

One of the advantages of the subject invention is its usefulness for detection of a wide range of target materials, binding events, and biochemical reactions amenable to analysis using the current inventive techniques. Many of these materials previously could not be detected using available assays. The present invention utilizes specific binding without the complications of immunoglobulin generation.

The rigor and outstanding advantages of the inventive assay system allow the detection and quantitative evaluation of materials which have been previously unachievable because of the limitations of the prior art methods. The present invention has already been tested in a unique assay method for accurate detection of malaria parasitic infection as described in Example 9. Development of an effective assay for malaria in transient stages has until now proven an intractable challenge for either the immunological assay or analytical chemical art methods.

IV. Qualitative and Quantitative Evaluation

In the subject invention and the assay, various spectral changes to the bi-layer are used to detect the presence or absence of the target material. The conjugated polymer backbone of the polymerized bilayer assembly signals binding at the surface of the film by a chromatic transition. The color or other spectral transition is readily visible to the naked eye as a blue to red color change and can be quantified by visible absorption spectroscopy.

The film was designed to undergo color transition from blue to red solely due to receptor-ligand interactions occurring at the surface of the bi-layer. The bi-layer assembly incorporated both a molecular recognition site and a detection element. This simple color-based sensor enables rapid, qualitative detection of binding by visual inspection of the film or quantitative detection by visible absorption spectroscopy.

As shown above, the thin films of chemically functionalized polydiacetylenes of the subject invention act

as simple colorimetric biosensors. These films are derivatized with a carbohydrate-based ligand which specifically binds bio-organisms such as viruses. The conjugated polymeric film is initially blue in color. Binding of a virus or other analyte to the derivatized polymer causes a change in color of the film from blue to red. The intensity of the resulting red color corresponds roughly to the quantity of the virus. Means of amplifying the spectral signal for quantification are well known in the art, such as scintillators, may advantageously be employed when low levels of analyte are present. Because of the empirical nature of the signal, there are many opportunities for automating the read out of the present inventive assay system.

In one embodiment of the present invention, a blue color shift is observed visually by the testing technician. Because of the simplicity of the reaction, such observation may easily be accomplished by an untrained person making the assay suitable, for example, for at-home testing. Alternatively, spectral test equipment, known in the art, may be employed to determine a change in spectral color-shift beyond the limits of simple visual observation, including optical density to a particular illuminating light wavelength.

Spectral changes outside the human visual range can be employed effectively in the subject invention by use of various spectral analyzers, such as light meters, or through technician observation of the surface using various translating devices, such as infrared and ultraviolet detectors.

Means of amplifying the spectral signal for quantification are well known in the art, such as scintillators, may advantageously be employed when low levels of analyte are present. Because of the empirical nature of the signal, there are many opportunities for automating the read out of the present inventive assay system.

For more precise quantitative measurement, the film is scanned with a visible absorption spectrometer where the relative change in the intensities at 620 nm (blue) and 550 nm (red) is readily assessed as seen in Figure 4 (Table 2). The

extent of the color response is directly proportional to the concentration of the analyte. The present example moves this technology from the realm of biodiagnostics to the realm of environmental diagnostics by exploring a new class of ligands for which a precedent exists for binding small organic molecules. These ligands are similarly tethered to the polydiacetylene backbone which provides the colorimetric detection.

UTILITY

The subject invention enjoys broad applications to the detection of a very wide variety of analytes. These include small biomolecules, the observation of binding and other chemical events, and the detection of trace amounts of many materials.

Because of the very broad applicability, important classes of analytes are detectable by the present invention which have previously proven difficult or impossible to detect by prior art methods. Many viruses, bacteria and proteins related to them, or infections caused by them can be detected. These include such pathogens as influenza, HIV, and malaria, among others. Direct colorimetric detection by the inventive polymeric films offers new possibilities of diagnostic application and screening for new drug candidates or binding ligands.

The present invention is useful for designer drug development and screening. Currently, to assess competitive inhibition of drug receptor molecules, radio-labeled materials are typically used. However, this process is time-consuming and requires access to and handling of radio-labeled materials. Other techniques, such as fluorescence quenching, are limited in that each test is self-contained, and therefore a large screening effort is prohibitively time consuming and expensive.

There are many advantages to the genetically conserved host recognition site being targeted by the embodiment of the present invention. For example, a determination of a patient's exposure to the flu will be definitive, and not limited to a particular strain if the binding to the influenza pathogen

receptor ligand is detected. This advantage of the present invention also avoids the need for a large number of immunological tests, as the clinician can rely on a single assay. Additionally, even newly evolved, uncharacterized flu strains can be identified, further avoiding false negative tests.

An analogous limitation of immunoassays occurs in well established pathogens such as malaria parasites. In these organisms, phases of the life cycle which would allow for an immune response have over time been so limited that the immune response is eliminated.

The present invention exploits the genetically conservative host binding site to identify the pathogen. Even in comparatively large parasites, the host binding site tends to be held constant over time throughout the generations of pathogens. Additionally, parasites are usually present in the body in a large number of diverse life stages. In well established parasites, the immune accessible sites often vary considerably from stage to stage, the advantage being that the host organism is unable to mount an immunological response with sufficient rapidity to avoid the entrenchment of the parasite.

In this particular application of the subject invention, various iterations of a drug can be quickly screened for interference with infective binding by a pathogen. Table 1 provides a number of examples of the host receptor molecules which provide the site of pathogen attachment required for infectivity. All of these examples, along with many others, can be exploited by the subject invention for drug development and optimization. Multiple wells which can be made on a single bi-layer sheet allow many subtle iterations of a candidate drug to be tested, such as various levels of pH titering. By using this invention for drug testing, the current chilling effect on drug research of expensive, individual testing for each sample would be eliminated.

The availability of high-volume inexpensive screening will dramatically increase the speed of drug development. For this purpose, a naturally occurring transmembrane receptor (TMR) is

reconstituted into a lipid bi-layer where the lipid layer is constructed from the polymerizable monomers. This is particularly applicable to the inventive compounds that have the two triple bonds in the chain. Once the receptor is incorporated into the lipid, the lipid is irradiated and polymerized to lock the TMR in place. Binding of small molecules to the binding site in the TMR produces a conformational change in the TMR which affects the lipids and causes a color change.

A wide variety of TMR's have been isolated. TMR's including hormone, neurotransmitter, and other physiological regulating receptors, are particularly useful in the improvement and development of drugs using the present invention. The use of naturally occurring TMR's in the subject invention has particular applications to drug screening. The subject invention has immediate pertinence in the development of new drugs of which biological effect depends on binding to membrane bound receptors. For example, the dopamine receptor binds the natural compound dopamine. In order to employ the subject invention to search for new compounds that behave like dopamine, that is, bind to the dopamine receptor, the dopamine receptor, used as a ligand, is exposed to the tested new dopamine-like drugs. The approach for drug testing is set out in Example 6 as one practical embodiment of the polymeric films of the invention and their applications.

Because of the ease of screening available using the subject invention, many small changes can be made in the candidate drug structure and analyzed immediately, providing great speed and flexibility in drug development and optimization. By noting the area of modification which provides the greatest changes in effectiveness, the critical structures of the drug can be rapidly identified. This allows a critical focusing of the drug modification effort which will greatly increase the speed of drug development.

Basic research of drug interactions, optimization, and new drug development is also made practical by the present invention. Existing drugs can be analyzed to determine which

structures are of the greatest importance in their therapeutic effect. These structures can then be optimized, and even transposed onto a more biologically acceptable, smaller, or less expensive non-active structure. Such qualities as the ability to traverse the blood-brain barrier can be conferred.

If two different drugs are available for the treatment of a disease, their structure can be analyzed as to activity using the technology of the subject invention. Then, their active sites can be incorporated into a single drug. Additionally, attendant structures which optimize activity can be appropriately situated on the new hybrid drug. Any interference in activity can be determined and ameliorated or eliminated prior to expensive and lengthy animal or human trials.

Another important application for the subject invention and method is the inexpensive, accurate assaying of infective states and other medical conditions. For instance, antibody levels to a specific pathogen can be easily and inexpensively monitored through competitive inhibition of a set amount of pathogenic material placed in the analytic solution. Additionally, certain antibodies can be detected through their direct and specific binding to the inventive membrane.

A large variety of biologically related materials is advantageously susceptible both to quantitative and qualitative analysis using the subject invention. Infection by various pathogens can be tested long before clinical manifestations are observed. This is of a particularly critical advantage in patients with depressed immunity, such as in newborns, chemotherapy patients, donor organ recipients, and AIDS victims.

In testing for pregnancy, human chorionic growth hormone is assayed using the present invention. A rise in luteinizing hormone heralds the onset of ovulation for both the achievement of pregnancy and for use in natural birth control methods.

Because of the simplicity of readout, the subject invention is highly suited for home use. For example, it enables multiple testing at low cost needed in natural birth

control methods or for assessing fertility to optimize the chances of achieving pregnancy.

The inexpensive multiple testing capacity of the present invention made possible through multiple wells on a single bi-layer sheet provides an excellent incentive for extremely early detection of pregnancy. Detecting pregnancy prior to a missed period is important in avoiding exposure to harmful factors, which are critical to the final outcome, in the first few days of pregnancy. It is also important when a pregnant woman may have been exposed to a disease that will have late or no clinical manifestation for the mother, but could severely damage the developing fetus she carries. These diseases can include rubella, toxoplasmosis, and other pathogens. The present invention allows for simple and inexpensive screening for such diseases.

Another important application for the present invention is the monitoring of patients with chronic illnesses such as diabetes. For instance, insulin blood levels can now be regularly monitored at home using the subject invention. This allows diabetics to tailor their insulin administration to more accurately follow the insulin requirements. It also allows them to quickly differentiate early symptoms of a transient illness such as flu from undue variations in insulin levels.

The present invention also allows for the production of a simple, at-home test for cholesterol levels allowing patients to determine their cholesterol levels in the privacy of their own home, encourages the more reticent to test their cholesterol level and be aware of this often critical information. For patients with known hypercholesterolemia, the present invention represents an ideal means to closely monitor the palliative effects of treatment efforts. The multiple well test kit made possible and practical by the present invention is particularly useful for weekly or even daily monitoring of these levels.

The monitoring of drugs and drug levels is a fertile area of application for the present invention. Patients typically display a wide range of metabolic levels and liver activity.

This is particularly the case for those in a hospital situation. Because blood drug levels cannot be easily determined, the clinician is often forced to under-medicate a patient who could benefit from higher levels of administration. Unfortunately, the doctor must err on the side of caution to avoid the possibility of toxic levels being reached. The present invention allows a more accurate titering of drug administration, allowing better pain relief and other drug benefits.

The present invention has important application in drug abuse. When a patient suffers from a possible overdose, the actual blood levels of the drug and also its identity can be very rapidly assessed by the treating physician using the present invention. This information prevents potentially harmful treatment for overdose by drugs which display the same symptoms as those of the actual overdose substance. Additionally, less draconian detoxification measures can be taken if lower than suspected drug levels are detected using the subject invention. Conversely, toxic levels can be detected even when the patient is not displaying symptoms which would alert the clinician to the actual danger level.

The subject invention is further useful in a wide variety of industrial applications. For instance, industrial enzymes can be monitored as to their binding strength, as well as to their presence in a media. Their loss can be monitored in an effluent, and their appropriate dispersal can be monitored in feedstock and media.

The invention is very useful in determining optimal conditions for enzyme activity on any particular substrate. Additionally, the enzyme can be easily engineered for optimization, including tailoring for specific uses or working environments. This is done in a manner analogous to designer drug evaluation as explained elsewhere. Thus, tolerance for extreme pH environments, concentrated feedstock, cold and heat, interfering additional materials, and other desirable tolerance can be developed for industrial enzymes and other active materials. The ability of the present inventive films to

detect small molecules using TMR's as described in the drug development section above also has excellent use in industrial and environmental applications. Noteworthy among TMR's to be used for this purpose are the olfactory TMR's. These can bind small odorant molecules and have important applications as an environmental sensor, among others.

The need for chemical sensors to measure analyte concentrations for industrial process control applications, for warning and safety systems, in environmental analysis, etc. is great. Classic chemical analysis, such as gas chromatography-mass spectrometry are not conducive to on-site field analysis because of the analytical turn around time, high cost, impractical equipment for field use and the need for technically experienced personnel. The sensor which would be useful in field work analysis therefore requires material which is chemically sensitive and can specifically bind the analyte in question, and a simple, user friendly method to detect when binding of the analyte has occurred. In-line monitoring of public water supplies (e.g. swimming pools, drinking water, waste water streams, etc.) for contaminants can be developed.

EXAMPLE 1

Preparation of Polymer Film For Detection of Influenza Virus

This example illustrates the procedure used for preparation of a polymeric film suitable for detection of influenza virus.

A polymerized bilayer assembly shown in Figure 1 composed of a self-assembled monolayer of octadecyltrichlorosilane (OTS) and a monolayer of functionalized polydiacetylene was prepared.

The films were prepared by a modified LB technique in which the carbohydrate ligand detection group is presented at the surface of the bi-layer. Mixtures of 2% to 5% of glycolipid monomer (13) seen in Figure 3 and matrix lipid monomer (11) were spread on the water surface of a standard LB trough.

The matrix lipid uniformly dispersed the sialoside lipid, which allowed optimum binding of the virus. Sialoside lipid 1% to 5% gave maximum binding of the virus to polymerized

liposomes. Ideal mixing of the two components was determined by analysis of the Langmuir isotherms. Various ratios of monomers (11) and (13) give isotherms whose limiting areas and collapse pressures change in direct proportion to the mole fraction of (2) as expected for miscibility. The mixed monolayer was compressed and polymerized on the water surface.

The floating polymerized film was lifted by the horizontal touch method onto a glass slide previously coated with a self--assembled monolayer of OTS. The resulting bi-layer assembly presents an array of carbohydrate ligands at the surface. The tetraethylene glycol spacer in sialoside lipid (13) serves to extend the carbohydrate ligand beyond the carboxylic acid head groups of the matrix lipid (11).

Films prepared in this manner exhibited a high degree of order over a macroscopic range (50 to 150 μM) as evidenced by optical microscopy with the use of crossed polarizers as shown in Figure 2. The films were further characterized by angle-resolved X-ray photoelectron spectroscopy (XPS) and ellipsometry. The XPS results indicate that the amide nitrogen atoms and the carbonyl carbon atoms of the head groups are localized at the surface relative to the methylene carbons of the lipid chains, demonstrating that the sialoside detection group is presented at the surface of the film. Ellipsometric analysis of the polydiacetylene monolayer coated on HF-treated silicon indicated a film thickness of ~40 Å, in agreement with the expected value based on molecular modeling. The bi-layer assembly had a visible absorption maximum of 620 nm and appeared as a blue film.

EXAMPLE 2

Qualitative Detection of Influenza Virus Bindings

This example illustrates the detection of the binding of influenza virus to the polymer film of Example 1.

When the film obtained in Example 1 was incubated with X31 influenza A virus in phosphate-buffered saline (PBS) buffer, at pH 7.41, the binding of the viral hemagglutinin to the sialic acid residues on the surface resulted in a blue to red color transition.

Figure (2)B shows the colorimetric response of the film, supported on a glass microscope slide, readily visible to the naked eye for qualitative evaluation of the presence of the virus. The film on the left (blue) has been exposed to a blank solution of PBS. The film on the right (red) has been exposed to 100 HAUs of virus (CR = 77%). A colorimetric response of ~15% was observed visually. No color change was observed when the blue film was incubated with a blank solution of PBS buffer. This result demonstrates a polydiacetylene color transition arising from affinity binding (affinitychromism) rather than thermal annealing (thermochromism).

Previous studies have shown that LB films composed of lipid (1), Reaction Scheme 1, undergo a blue to red color change when heated at 70°C, which corresponds to the endothermal transition for lipid chain melting. Lipid chain disorder and tangling decrease the effective conjugation length of the polydiacetylene backbone. Similarly, Fourier transform infrared and resonance Raman spectroscopy as well as X-ray data demonstrate that lipid chain packing of the red form of the polymer is different from that of the blue form. Thus, conformational changes in the lipid chains affect the optical properties of the polymer backbone. Binding of the viral hemagglutinin to the sialoside bi-layer assembly appears to affect the lipid chain conformations in a manner analogous to thermal annealing.

EXAMPLE 3

Quantitative Detection of Influenza Virus Binding

This example describes the quantitative detection of binding of influenza virus to the polymeric film of the invention.

In addition to qualitative evaluation by visual inspection, the degree of color change is readily quantified by standard visible absorption spectroscopy. The visible absorption spectra of a bi-layer assembly prior to (solid line) and after (dashed line) viral incubation are shown in Figure 4. The bi-layer assembly was inserted into a quartz cuvette containing PBS buffer (pH 7.4), and the absorption spectrum was

obtained. Addition of influenza virus in PBS buffer (pH 7.4) resulted in a chromatic transition following a 30-min. incubation period. Although the film color began to change within seconds after exposure to virus, 30 min. was found to be the average length of time required for the CR to reach a plateau value in a nonstirred solution. These spectra represent a CR of 50%.

The blue-colored film (solid line) before the exposure to the virus, had a strong absorption maximum at 620 nm and a weaker absorption at 550 nm. After incubation with influenza virus (dashed line), a dramatic change in the visible absorption spectrum occurred. The maximum at 550 nm increased with a concurrent decrease in the maximum at 620 nm, resulting in a red-colored film.

In order to quantify the response of a film to a given amount of virus, the visible spectrum of the film before exposure to virus was analyzed as equation

1. $$B_o = I_{620}/(I_{550}+I_{620})$$

where B_o is defined as the intensity of absorption at 620 nm divided by the sum of the absorption intensities at 550 nm and 620 nm. After exposure to influenza, the equation was

2. $$B_v = I_{620}/(I_{550}+I_{620})$$

where B_v represents the new ratio of absorption intensities after incubation with the virus. The colorimetric response (CR) of a film is defined as the percent change in B upon exposure to virus:

3. $$CR = [\ (b_o-B_v)/B_o)]\ \cdot\ 100\%$$

The colorimetric response was directly proportional to the quantity of influenza virus, measured in hemagglutinating units (HAUs), where 1 HAU is defined as the highest dilution of stock virus that completely agglutinates a standard erythrocyte suspension.

Figure 5 shows a plot of the colorimetric response of a sialoside bi-layer assembly versus successive additions of influenza virus. A blue film containing 2% of sialoside lipid 13 (Figure 3) and 98% matrix lipid 11 (Figure 3) was preincubated in PBS buffer for 30 min., after which successive

aliquots of X31 influenza A virus were added. The film was incubated for 30 min. following each addition of virus, and the visible absorption spectrum was recorded. The CR was calculated according to equation 3. Linear regression analysis of the first six data points gives a slope of 0.93% (r^2 = 0.985).

Saturation of the colorimetric response occurs at ~80 HAUs. Incubating the red film with a buffer blank (no virus) for 1 hour did not result in a return of the blue color. Thus, the structural changes which result in the color change appear to be irreversible under these conditions.

EXAMPLE 4

Competitive Inhibition Assays For Influenza Virus Specific Detection

This example illustrates utility of the invention in competitive inhibition assays.

The specific nature of the interaction between the influenza virus and the sialoside film surface was confirmed by competitive inhibition assays. Figure 6 shows that the CR of the film can be inhibited by compounds that bind to viral hemagglutinin. Incubation of a sialoside bi-layer assembly with 32 HAUs of influenza virus produced a colorimetric response of 22.6%. However, the same concentration of virus in the presence of 17.3 mM concentration of compound 17 (K_d = 2 mM) (Figure 6, column 4) completely suppressed the CR to less than 0.5% due to competitive inhibition. The CR was not diminished in the presence of 17.3 mM (Figure 6, column 5) concentration of compound 19 (K_d > 50 mM) or compound 21 (Figure 6, column 6) that do not compete for binding to viral hemagglutinin. The known inhibitor of influenza hemagglutination, compound 17 see in Figure 3, has dissociation constant K_d of 2 mM as determined by a standard hemagglutination inhibition assay (HAI). Incubation of the sialoside bi-layer assembly with influenza virus in the presence of the known binding inhibitor 17 resulted in no CR (CR < 0.5%) and the film remained blue. This result demonstrates that the inhibitor effectively competed with the

sialoside surface for binding to the virus. When the blue film was exposed to the same quantity of influenza in the presence of a noninhibitor (Figure 3, compound 19, Kd > 50 mM, or glucose, compound 21), the color change was identical to a film exposed to influenza alone.

In order to test the capability of the film to predict the value of Kd for an inhibitor, the CR was measured for a series of inhibitor concentrations. The CR increased in a linear fashion (r^2 = 0.995) with decreasing concentrations of inhibitor 17. Extrapolation of this plot to CR = 0% gives the inhibitor concentration that completely prevents binding of the virus to the surface. This value represents the minimum inhibitor concentration required to effectively compete with the sialoside surface. The value obtained, 2.5 ± 0.83 mM per 4 HAUs of virus, is in agreement with the value of 2 ± 1.1 mM obtained by a standard HAI assay and 2.8 ± 0.30 mM as obtained by nuclear magnetic resonance spectroscopy.

The inventive inhibition assay described here is direct and easy to perform. This approach avoids the need for red blood cells, which are used in the standard HAI assay. In addition, the subjectivity of reading microliter plates in the standard HAI assay is replaced by a quantitative spectrophotometric method. This methodology could be applied to screening for new drug candidates or binding ligands.

EXAMPLE 5

Non-specific Adhesion

This example illustrates the non-existence of non-specific adhesion interfering with the assay of the invention.

In order to assess the CR due to non-specific adhesion, two experiments were performed. In the first experiment, films incorporating lactose lipid (15) (Figure 3) were incubated with the influenza virus. Lactose was not a ligand for the hemagglutinin lectin. Incubation with 100 HAUs of the virus, which is a concentration corresponding to a maximum response in the sialoside films, show only a small effect (CR of 2 to 4%). In the second experiment, films containing sialoside lipid 13 were exposed to concentrated solutions of bovine serum

albumin. Again, the same small CR was observed. These results indicate that non-specific adhesion of virus or protein to the film surface does not produce the dramatic color change observed from specific receptor-ligand binding.

EXAMPLE 6

Drug Development

This example illustrates the utility of the invention for drug development.

A receptor and its reciprocal binding partner (receptor-binding molecule) known to be involved in the physiological regulation of interest were selected. The binding partner was incorporated into the inventive film according to Example 1.

In the case of neurotransmission and neurological drug development, for instance, a dopamine receptor was employed. In the case of drug development toward pathogens such as influenza, for instance, the viruses hemagglutinin receptor were employed. The binding partner incorporated into the film was dopamine, or a dopamine analog or sialic acid or sialic acid analog, respectively. The goal of the assay was to select a drug which interacts with the binding site in a way which effects physiological functions.

When the described receptor was present, it caused a color change when allowed to bind to the inventive membrane incorporating the binding partner.

A candidate drug was then introduced into the system. If the drug bound to the receptor or modified the binding partner's binding capacity, there was a concomitant decrease in the color change observed in the subject inventive membrane due to competitive inhibition. The ability of the candidate drug to influence binding was quantitated by observing the degree of decrease in signal as compared to the control.

Variations of the above approach are used to suit different systems. In some cases it is necessary to organize the receptors into large assemblages such as incorporation into polymers, liposomes or membranes. This arrangement amplifies the film changes when the receptors bind. However, when a candidate drug is introduced which binds the receptor as above,

there is a concomitant decrease in the observed color change.

Another variation that is applicable to this invention requires that the receptor portion be attached to the film. A known receptor is covalently attached to the film at one or more points. This can be accomplished by appending the receptor to the monomer prior to film polymerization (as in the foregoing example for binding partners) or after the film is polymerized through modification of the film's surface. Binding partners then interact with the immobilized receptors distorting the film, giving rise to the color change. In this way, test candidates can be directly screened with receptors giving a positive film response rather than absence of color change, as in the previous variation.

EXAMPLE 7

Generalized Approach

This example illustrates general utility of the invention.

Generic film is made where the binding partner is kept constant and an intermediate linking moiety is varied to accommodate an ancillary novel binding partner.

A film which has biotin on its surface binds the protein streptavidin. This protein is tetravalent, therefore in its bound state it still has one or more sites available for biotin binding. Novel binding partners are derivatized with biotin to attach themselves to the film surface through the intermediacy of the streptavidin protein. Only a single biotinylated film is prepared, and built up in a sandwich fashion with streptavidin, the biotinylated test binding partner. Exposure of this assembly to the test receptor gives the desired color change. As in the previous example competitive assays are performed to identify new drug candidates.

EXAMPLE 8

Entrapment and Detection of Small Organic Molecules

This example shows the development of a new class of functional materials which specifically trap small organic compounds and report the entrapment event by a colorimetric change which can be detected visually. These materials act as

simple color-based sensor devices which detect the presence of compounds such as solvents or other toxic pollutants in air or water streams.

The first step involves the synthesis of lipid diacetylene analogs of compounds 1 and 2 a seen in Reaction Scheme 1 by elaborating the secondary methyl group into the orientation group. The enantiometrically pure ester of pentacosadiynoic acid 3 (PDA) is hydroxylated via molydenum peroxide oxidation to alcohol 4. Diasteriomers are separated and the ester is hydrolyzed to chiral lactate analogs 5 and 6. The ethyl esters are formed and treated with Grignard reagents to give the desired chiral lipid analogs 7 and 8. Variation in the R groups result in a wide variety of new materials in which the specific entrapment capabilities are reviewed.

The monomer-lipid clathrate is ordered and compressed on the water surface using a Langmuir-Blodgett film apparatus. Polymerization of-the monolayer by UV irradiation yields the blue colored material as previously described. The is lifted onto a hydrophobized microscope slide. The ability of films of 7 and 8 to entrap dioxane and 1-butanol and to undergo the expected color transition was tested. Because the technique can be generalized, appropriate derivatives of 1 and 2 are selected and tuning of the chemistry to specifically detect a particular small molecule is determined. To date, little is known about why the materials 1 and 2 are highly selective to dioxane and 1-butanol, respectively. By examining a series of compounds, a variety of solvents was screened using the colorimetric detection technique to determine which solvent forms the most suitable guest compound. By using computer modeling, cavities were engineered to be of the specific size and shape to bind to analyte molecules. The uncomplexed and complexed film with a variety of standard surface techniques were fully characterized. These include XPS, Auger, Leed, ellipsometry, Raman spectroscopy and STM. All of these enabled determination of the structural requirements of the clathrates in order to rationally design new materials with specific clathration properties.

EXAMPLE 9

Detection of Malaria Merozoites

This example describes the films and conditions used for detection of malaria merozoites.

The films contained sialic acid and were prepared identically to those described in Example 1. The films were exposed to erythrocyte containing solutions of malaria merozoites. After overnight exposure to the pathogens the films became pink in color. The color response (CR) in each case was nearly 100%.

TABLE 1

Pathogen	Receptor Molecule
HIV	D4[4]; Vasoactive Intestinal Peptide[5], Peptide T[6], Sialic Acid[7]
Vaccinia	Epidermal Growth Factor[8]
Rabies	Acetylcholine receptor[9]
Epstein Barr	Complement Receptor[10]
Rheo	Beta-adrenergic receptor[11]
Rhinovirus	ICAM-1[12,13]; N-CAM, myelin-associated glycoprotein MAb[14]
Polio viruses	Polio viruse receptor[15]
Influenza	Sialic Acid[16]
Cytomegalovirus	Glycoprotein (not Sialic Acid)[16,17,18]
Coronaviruses	9-OAC Sialic Acid & Sialic Acid
Encephalomyelitis	9-OAC Sialic Acid
Rubella Virus	----------[19]
Measles Virus	Glycoprotein (not Sialic Acid)[20,21,22,23]
Herpes	Oligosasaccharioes Glycoprotein[24,25,26]
Chlamydia	Sialic Acid[27,28,29,30]
Rhinovirus	Glycosylated Proteins[31,32]
Rotavirus	9-OAC Sialic Acid
Polyomavirus	Sialic Acid
Reovirus	Sialic Acid
Streptococcus Suis	Sialic Acid α 2 → 3 Poy-N-Acetyllactosamine
Salmonella Typhimurium	Sialic Acid
Paramyxovirus	
Sendi Virus	Sialic Acid
Humps	Sialic Acid
Newcastle Disease Virus	Sialic Acid
Myxoviruses	Sialic Acid
Escherichia Coli	Oligomannose, Galactose α 1 → 4 Galactose, Sialic Acid α 2 → 3 Galactose
Encephalomyocarditis Virus	Sialic Acid
Cholera Toxin	G_{M1} (A Gangliosial of Sialic Acid, Galactose, Glucose, N-Acetyl Galactos)
Meningitis	Sialic Acid

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2. Science, 215: 182 (1982)
3. Proc. Natl. Acad. Sci. USA, 81: 4510 (1984)
4. J. of Biol. Chem., 265: 12293 (1990)
5. Proc. Natl. Acad. Sci. USA, 82: 1494 (1985)
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7. J. of Neuroscience Research, 18: 102-107 (1987)
8. FEBS Letters, 211: 17-22 (1987)
9. Cell, 56: 855-865 (1989)
10. Cell, 56: 839-842 (1989)
11. Cell, 56: 849-853 (1989)
12. Nature, 333: 426-431 (1988)
13. Proc. Natl. Acad. Science, USA, 85: 7743-47 (1988)
14. Nature, 312: 763-770 (1985)
15. Cell, 56: 725-728 (1989)
16. J. Virol, 63: 3991 (1989)
17. Inas, 86: 10100 (1989)
18. Virol, 176: 337 (1990)
19. Med. Microbio. Imm., 179: 105 (1990)
20. Infect. Imm., 24: 65 (1979)
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25. Science, 248: 1410 (1990)
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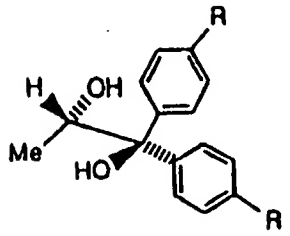
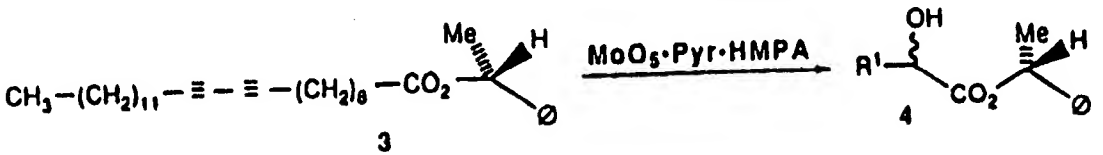
30. Infect. Imm., 25: 940 (1983)
31. Med. Virol, 8: 213 (1989)
32. J. Virol, 64: 2582 (1990)

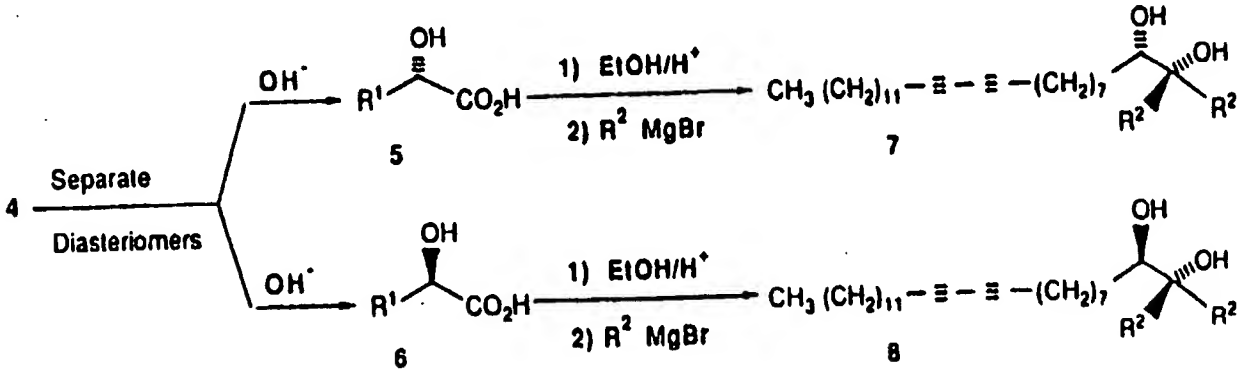
REACTION SCHEME 1

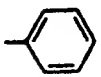


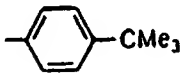
1: R = H
2: R = CMe_3



$\left(R^1 = CH_3(CH_2)_{11}-\equiv-\equiv-(CH_2)_7-\right)$



a: R^2 = 

b: R^2 = 

CLAIMS

1. A polymerized bi-layer film for direct detection of the presence of analyte comprising:

a) a ligand comprising detecting group having direct affinity for the analyte or functions as a competitive binder to the analyte;

b) a linear structural linker having two terminal ends, wherein said linker is attached at its first terminal end to said ligand;

c) a conjugated polymer backbone to which said structural linker is bound at its second terminal end;

d) orienting groups which are bound to the surface of the conjugated polymer backbone in positions not occupied by the structural linker; and

e) a support structure;

wherein said film undergoes detectable spectral modification upon binding of a target analyte to the ligand.

2. The film of Claim 1, wherein the analytes are biomedical materials, pathogens, drugs, or industrial materials.

3. The film of Claim 2, wherein said biomedical materials are selected from the group comprising pathogens and cells infected by them, drugs, hormones, blood components, disease indicators, cell components, antibodies, lectins, enzymes, genetic material, and their metabolic derivatives.

4. The film of Claim 2, wherein said pathogens are selected from the group comprising viruses, bacteria, parasites and other pathogens.

5. The film of Claim 4, wherein said virus is selected from the group comprising influenza, cold, rubella, chicken pox, hepatitis A, hepatitis B, herpes simplex, polio, small pox, plague, HIV, vaccinia, rabies, Epstein Barr, reovirus, rhinovirus, and mutations, strains and/or ligand recognizable parts thereof.

6. The film of Claim 4, wherein said bacteria are selected from the group comprising E. coli, tuberculosis, salmonella, streptococcus, and mutations, strains and degraded

parts thereof.

7. The film of Claim 4, wherein said parasites and other pathogens are selected from the group comprising malaria, sleeping sickness, river blindness, and toxoplasmosis.

8. The film of Claim 1, wherein said ligand is provided for the detection of a pathogen analyte.

9. The film of Claim 8, wherein the analyte is a virus.

10. The film of Claim 8, where said ligand is selected from the group comprising epidermal growth factor for vaccinia analyte, acetylcholine receptor for rabies analyte, complement receptor for Epstein Barr analyte, beta-adrenergic receptor for reovirus analyte, ICAM-1 for Rhinovirus analyte, polio virus receptor for polio virus analyte, trisaccharide analyte for cholera toxin analyte, tetrasaccharide for neutrophil analyte, and derivatives and analogues thereof capable of associating with analyte.

11. The film of Claim 8, wherein said ligand is sialic acid and its derivatives and analogs which bind to corona virus, influenza virus, encephalomyelitis virus, chlamydia, sendi virus, mumps virus, newcastle disease pathogen, myxovirus, encephalo-myocarditis virus, meningitis virus, or malaria virus.

12. The film of Claim 8, wherein the ligand and analyte pairs are tetrasaccharides and neutrophiles, cell adhesion peptides and target cells, trisaccharides and bacterial toxins, or transmembrane receptors and hormones.

13. The film of Claim 8, wherein the ligand provided to detect HIV analytes is selected from the group comprising CD4, sCD4, CD26, vasoactive intestinal peptide, peptide T, and sialic acid, and derivatives and analogues thereof capable of associating with HIV.

14. The film of Claim 1, wherein said polymer comprises of polymerizable lipid monomers.

15. The film of Claim 14, wherein said monomer is selected from the group comprising acetylenes, diacetylenes, alkenes, thiophenes, imides, acrylamides, methacrylates, vinylether, malic anhydride, urethanes, allylamines, siloxanes,

anilines, pyrroles and vinylpyridinium.

16. The film of Claim 15, wherein said polymer backbone is comprised of diacetylene monomers.

17. The film of Claim 1, wherein said orienting head groups are hydrophilic, with the capacity to mutually form a hydrogen bond.

18. The film of Claim 17, wherein said orienting head groups are selected from the group consisting of $-CH_2OH$, $-CH_2OCONHPh$, $-CH_2OCONHEt$, $-CH_2CH(Et)OCONHPh$, $-(CH_2)_9OH$, $-CH_2OCOPh$, $-CH_2OCONHMe$, $-CH_2OTs$, $-CH(OH)Me$;

$-CH_2OCOR_2$, wherein R_2 is $n-C_5H_{11}$, $n-C_7H_{15}$, $n-C_9H_{19}$, $n-C_{11}H_{23}$, $n-C_{13}H_{27}$, $n-C_{15}H_{31}$, $n-C_{17}H_{35}$, Ph, PhO, or $o-(HO_2C)C_6H_4$;

$-OSO_2R_2$, wherein R_2 is Ph, $p-MeC_6H_4$, $p-FC_6H_4$, $P-ClC_6H_4$, $p-BrC_6H_4$, $p-MeOC_6H_4$, $m-CF_3C_6H_4$, $2-C_{10}H_7$, or Me;

$-CO_2M$, wherein M is K, HNA, or Ba/2; or

$-CH_2OCONHR_2$ or $-CH_2CONHR_2$ where R_2 is Et, n-Bu, $n-C_6H_{13}$, $n-C_8H_{17}$, $n-C_{12}H_{25}$, cyclo-C_6H_{11}, Ph, $p-MeC_6H_4$, $m-MeC_6H_4$, $o-ClC_6H_4$, $m-ClC_6H_4$, $P-ClC_6H_4$, $o-MeOC_6H_4$, 3-Thienyl, Me, Et, Ph, $1-C_{10}H_7$, Et, Ph, $EtOCOCH_2$, $BuOCOCH_2$, Me, Et, i-Pr, $n-C_6H_{13}$, $EtOCOCH_2$, $BuOCOCH_2$, Ph, $2,4(NO_2)_2C_6H_3OCH_2$, CH_2CH_2OH.

19. The film of Claim 17, wherein said orienting head group is a carboxylic acid.

20. The film of Claim 1, wherein the orienting group of the monomers is selected from the group CH_3-, CH_3O-, neo-$C_5H_{11}O-$, cyclo-$C_6H_{11}O-$, $PhCH_2O-$, $p-AcC_6H_4O-$, $p-BzC_6H_4O-$, $pBrC_6H_4COCH_2O-$, $p-(PhCH=CHCO)C_6H_4O-$, $p-(PhCOCH=CH)C_6H_4O-$, $o-BZC_6H_4NH-$, $p-BZC_6H_4NH-$, $MeOCH_2CH_2NH-$, $n-C_6H_{13}NH-$, or EtO-. 21. The film of Claim 20, wherein the terminal is a methyl group.

22. The film of Claim 1, wherein the film is additionally provided with a support.

23. The film of Claim 22, wherein the support is selected from the group comprising plastic, mica, metal, ceramic, glass, and other polymeric surfaces and hydrophobic derivatives thereof.

24. The film of Claim 23, wherein the support is a hydrophobized glass slide.

25. A test kit comprising a container incorporating the

film of Claim 1.

26. The test kit of Claim 25, wherein a solid structure provided with orifices is fitted on the surface of the film to provide well structures which are analyte fluid impermeable.

27. The test kit of Claim 25, wherein said kit container also incorporates instructions as to implementation of the test procedure.

28. An analytical device incorporating the film of Claim 1.

29. The analytical device of Claim 28, wherein said device is provided with an automated chromatographic read-out component.

30. The analytical device of Claim 29, wherein said device is provided with an automated sample handling component to allow continuous readings on a flow stream or a series of samples.

31. A method of making the polymerized bi-layer assay film of Claim 1, comprising the steps of:

a) attaching a receptor binding ligand to a first terminal end of linear structural unit;

b) attaching a monomer to a second terminal end of the linear structural unit to produce a monomer-linear structural unit ligand moiety;

c) attaching an orienting head group to monomers, producing an orienting head group-monomer moiety;

d) mixing said monomer-linear structural unit-ligand moiety with a plurality of orienting head group-monomer moieties;

e) spreading the mixture of step d) on a surface; and

f) polymerizing said spread mixture.

32. The method of Claim 31, additionally comprising the step of transferring the polymerized mixture to a support.

33. The method of Claim 31, wherein after the step of e) said spread mixture is compressed.

34. The method of Claim 31, wherein after the step d) said mixture is spread on a water surface.

35. A method for the direct detection of an analyte,

said method comprising steps:

a) contacting the polymerized bi-layer assay film of Claim 1 with a sample which contains an analyte, and

b) assessing the film for a change in its optical properties.

36. The method of Claim 35, wherein the optical property observed is a change in color.

37. The method of Claim 36, wherein the color change is from blue to red.

38. The method of Claim 37, wherein the color change is detectable at the wavelength from 620 nm to 550 nm.

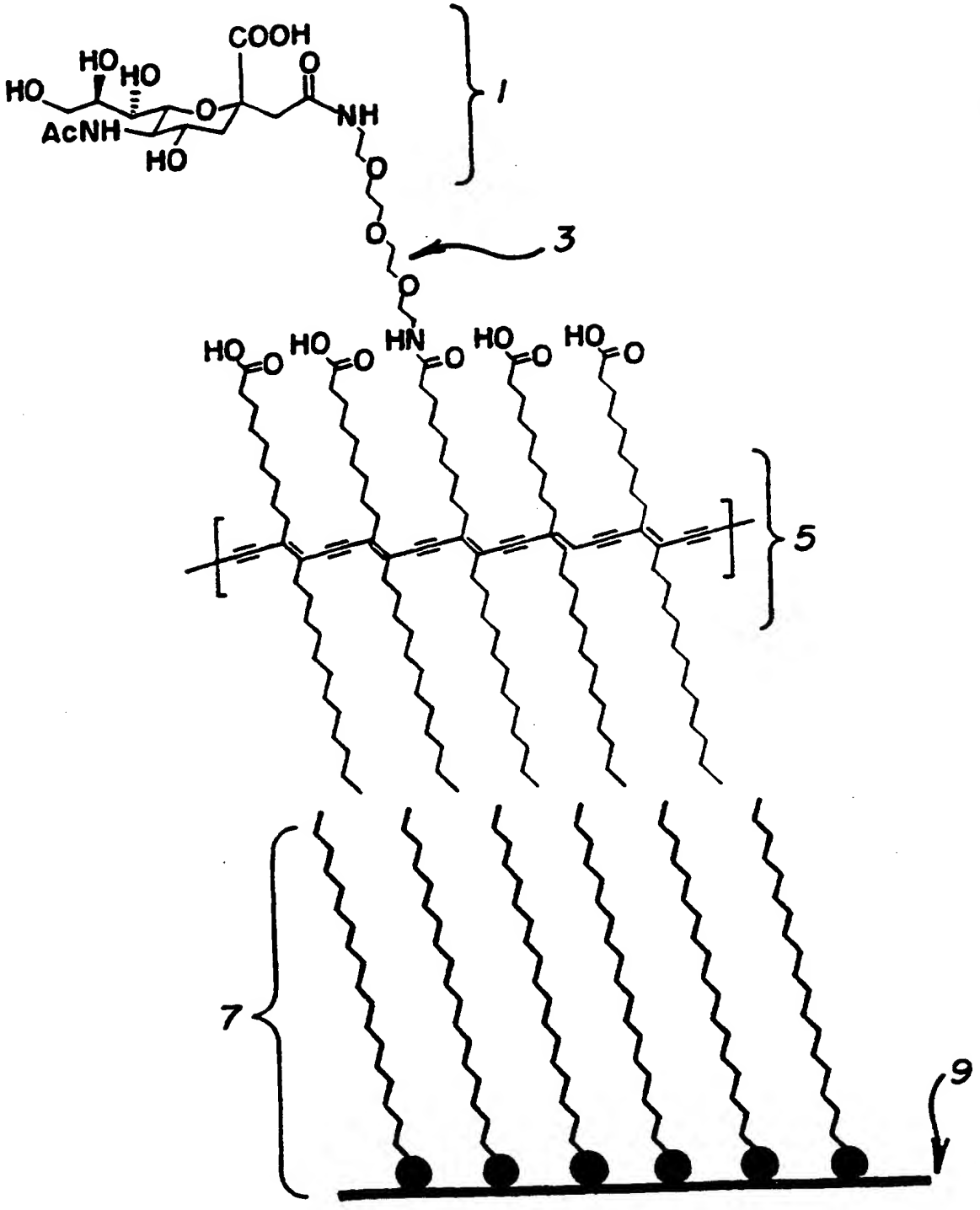


FIG. 1

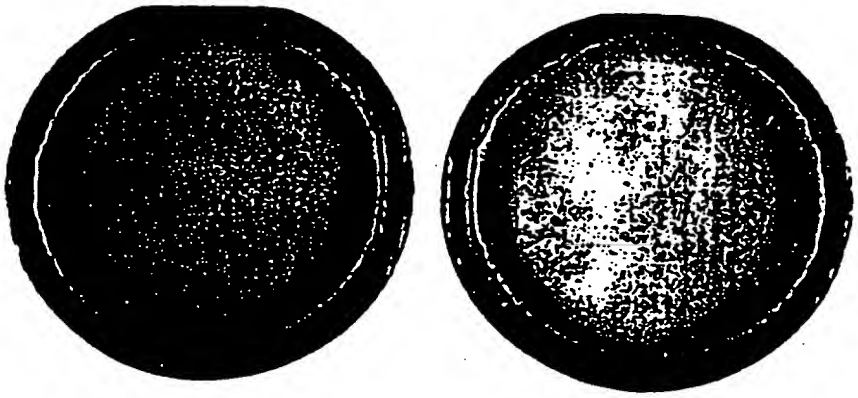


FIG. 2B

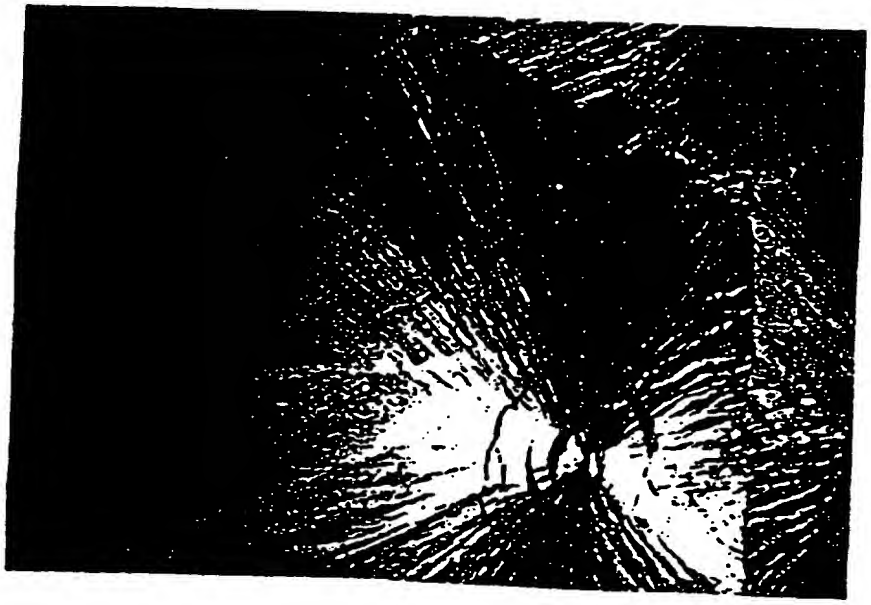


FIG. 2A

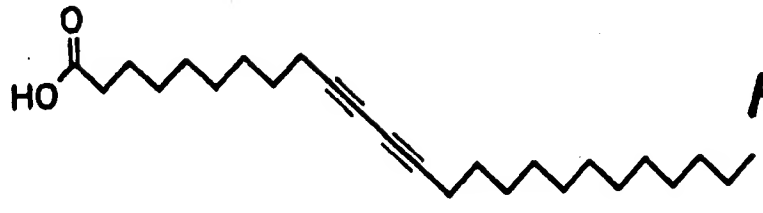


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FIG. 3A

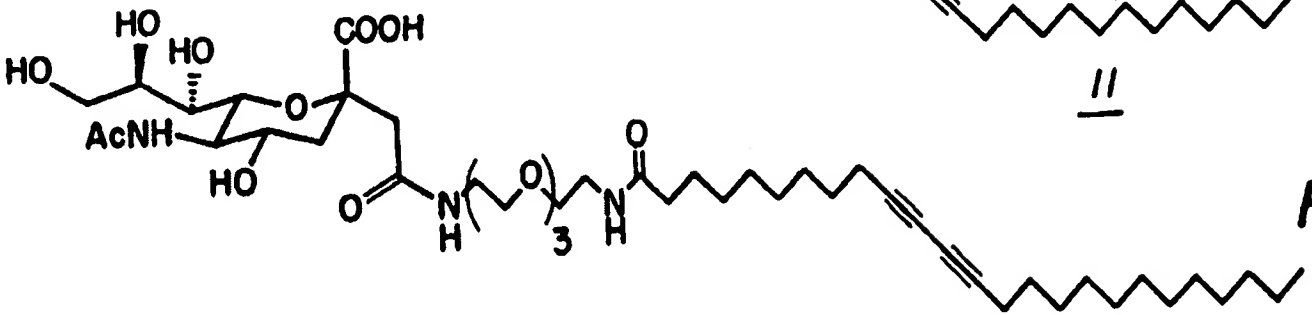

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FIG. 3B

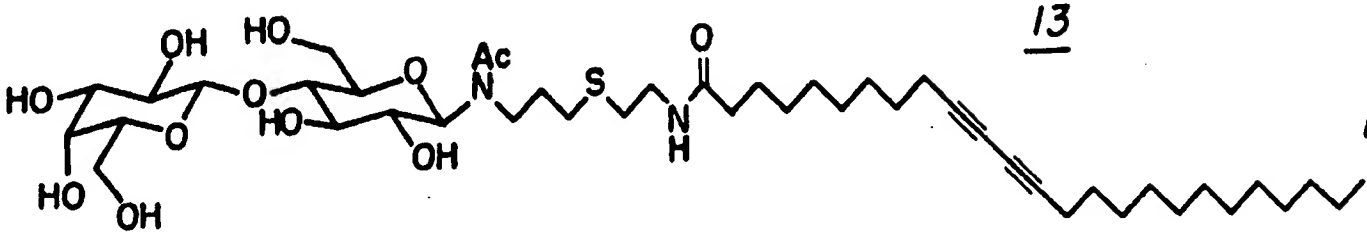

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FIG. 3C

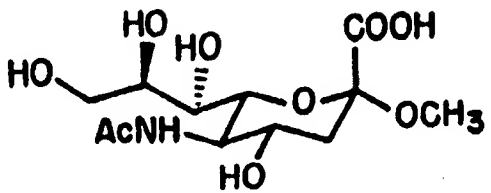

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FIG. 3D

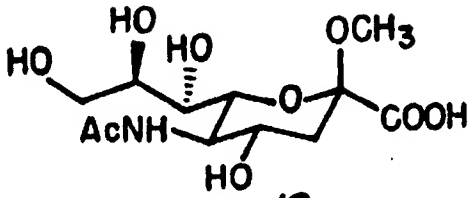

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FIG. 3E

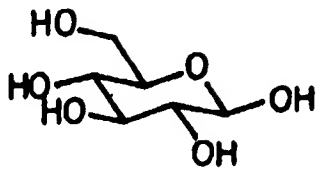

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FIG. 3F

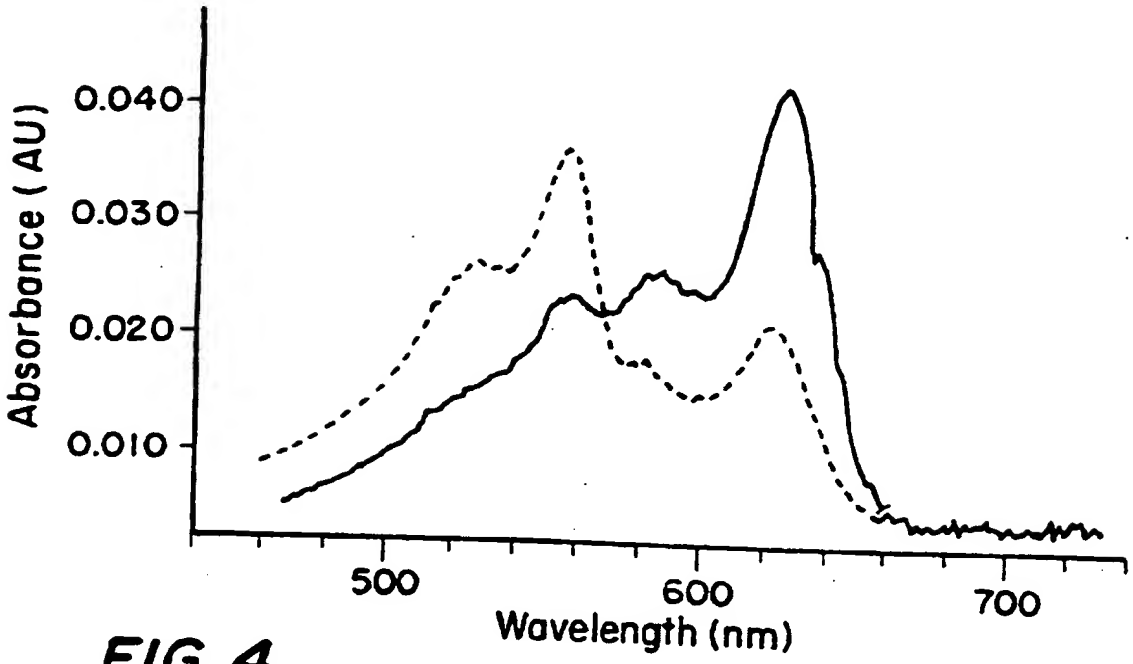
Absorbance (AU)
0.040
0.030
0.020
0.010
500
600
700
Wavelength (nm)

FIG. 4

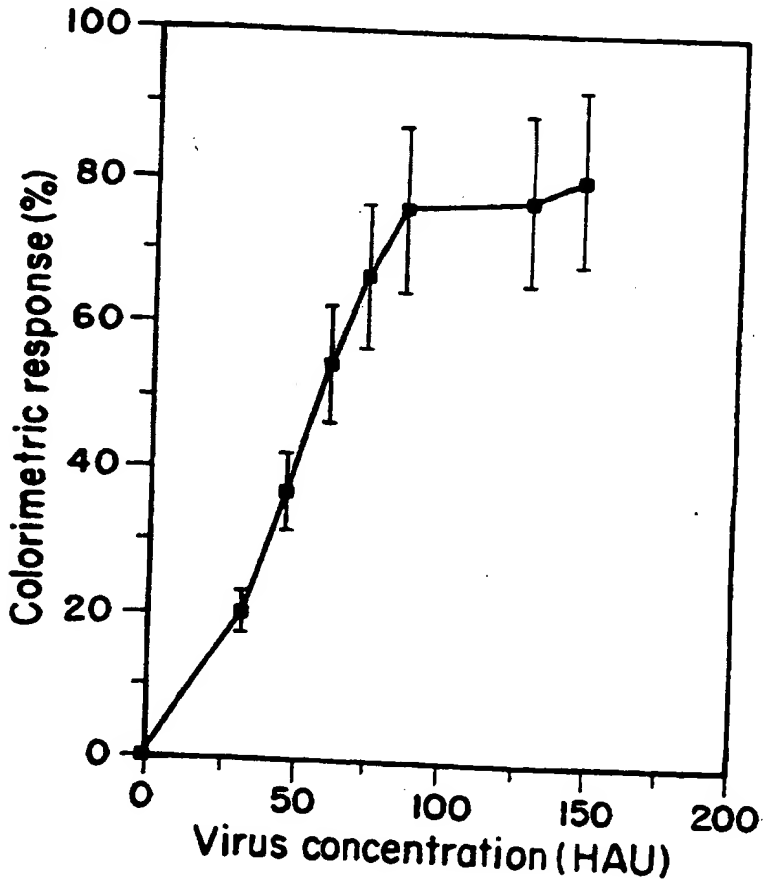
Colorimetric response (%)
100
80
60
40
20
0
0
50
100
150
200
Virus concentration (HAU)

FIG. 5

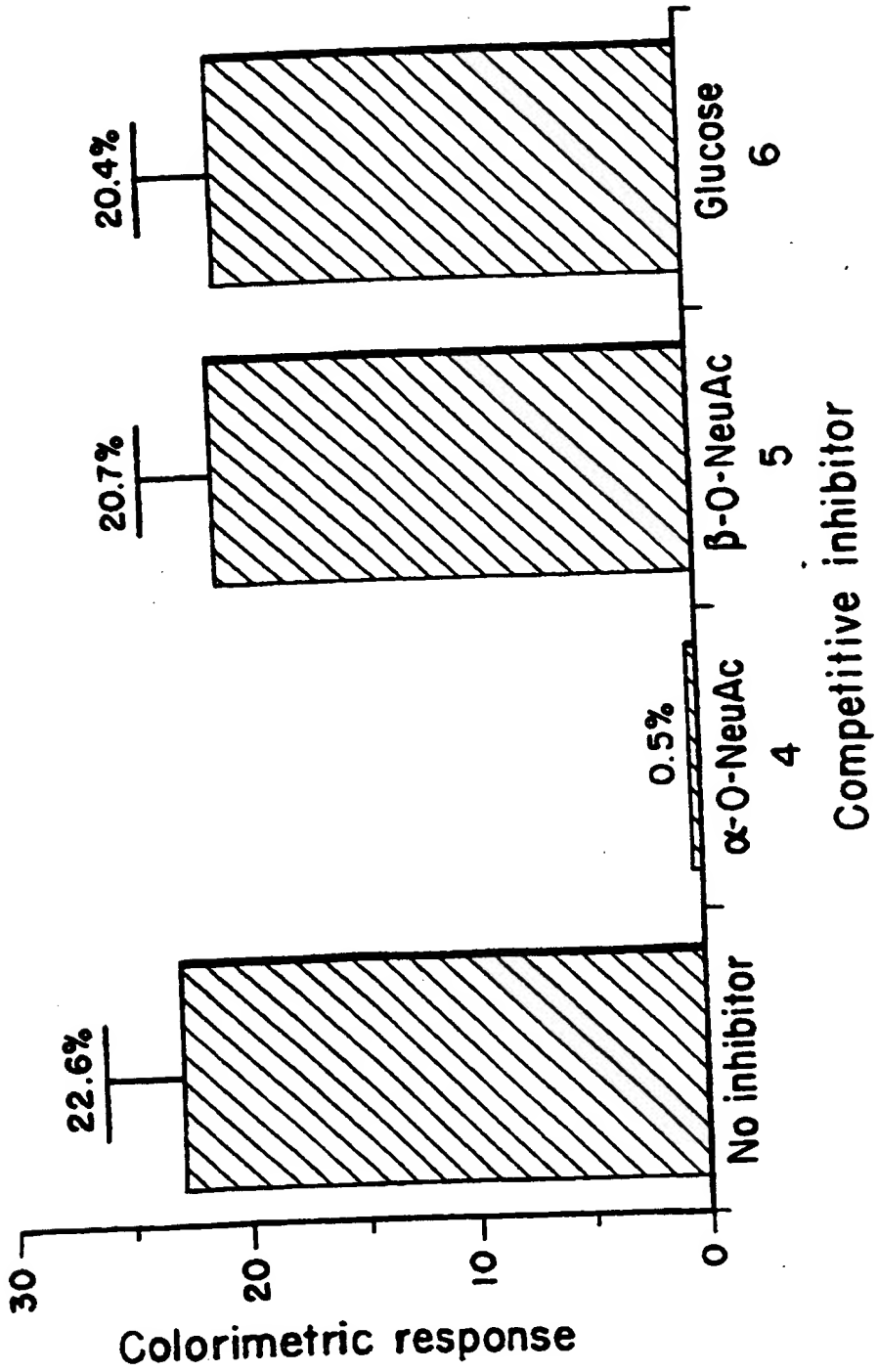

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01291

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/00; G01N 33/53, 33/92, 21/03
US CL : 435/4, 7.1; 436/71, 165, 807

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.1; 436/71, 165, 807

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DAILOG, APS. author and word search. search terms: film, assay, bilayer, polymer?, lipid?, linker?, ligand, analyte, optical, spectroscop?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	UCHIDA et al. Control of Surface Chemical Structure and Functional Property of Langmuir-Blodgett Film Composed of New Polymerizable Amphiphile with a Sodium Sulfonate. Macriomolecules. 1991, Vol. 24, No. 11, see entire document.	1-38
Y	CHARYCH et al. Specific Interaction of Influenza Virus with Organized Assemblies of Polydiacetylenes. Materials Research Society Symposium Prceedings. 1993, Vol. 293, "Biomolecular Materials", pages 153-161, see entire document.	1-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 MARCH 1997	Date of mailing of the international search report 07 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DW for HEATHER BAKALYAR Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01291

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OYAMA et al. A Redox-Active Polymer Film Mediated Enzyme Electrode for Amperometric Determination of Free Cholesteral. Electroanalysis. 1991, Vol. 3, pages 665-671, see entire document.	1-38
Y	CHARYCH et al. Self-Assembled and Langmuir-Blodgett Organic Thin Films As Functional Materials. MRS Bulletin. Vol. 17, No. 11, November 1992.	1-38
Y	LITVIN et al. Langmuir films of amino acid-modified diacetylenes as organic templates for biomimetic mineralization. Proceeding SPIE-The International Scoiety for Optical Engineering. 1995, Vol. 2441, pages 54-60, see entire document.	1-38
Y	WILSON et al. Enzymatic Modification and X-ray Photoelectron Spectroscopy Analysis of a Functionalized Polydiacetylene Thin Film. Langmuir. 1994, Vol. 10, pages 1512-1516, see entire document.	1-38

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